

ANALYSIS OF TRANSCRIPTS EXPRESSED BY *EIMERIA TENELLA* OOCYSTS USING SUBTRACTIVE HYBRIDIZATION METHODS

K. B. Miska, R. H. Fetterer, and R. C. Barfield

USDA-ARS, Animal Parasitic Diseases Laboratory, 10300 Baltimore Avenue, Building 1042 BARC-East, Beltsville, Maryland 20705. e-mail: kmiska@anri.barc.usda.gov

ABSTRACT: To characterize the genes expressed by *Eimeria tenella* oocysts, the sequence of 499 expressed sequence tags (ESTs) was obtained from complementary DNA (cDNAs) enriched for transcripts expressed by unsporulated or sporulated oocysts. Of these, 225 clones were isolated from cDNA of sporulated oocysts and 274 from unsporulated oocysts. A total of 163 unique sequences were found, and the majority of these (64%) represent novel genes with no significant homology to the proteins in GenBank. Approximately half of the unique transcripts generated from sporulated oocysts are also expressed by sporozoites and merozoites, whereas the expression of most (79%) of the transcripts from unsporulated oocysts has not yet been detected at other stages of development. The expression of 4 transcripts obtained from the subtracted cDNAs was confirmed by quantitative reverse transcriptase–polymerase chain reaction. The results confirmed that these transcripts are in fact differentially expressed between sporulated and unsporulated oocysts.

The protozoan parasite *Eimeria tenella* is 1 of the 7 species of *Eimeria* that cause coccidiosis in chickens. The pathology of this disease is produced by rapid replication of the parasite in the cells of the intestinal epithelium, resulting in diarrhea, weight loss, and anorexia. This condition causes significant economic losses to the poultry industry worldwide, with over US\$600 million being spent per year in the United States alone (Allen and Fetterer, 2002). New approaches in treating coccidiosis are continually being sought because resistance to anti-coccidials is ever increasing (Martin et al., 1997). Development of novel vaccines and drug targets hinges on the discovery of appropriate parasite gene products to serve as targets for these therapies.

The life cycles of *Eimeria* are complex, and it is likely that gene expression profiles in coccidia differ significantly between developmental stages. In the past, sequencing expressed sequence tags (ESTs) has been an effective method of finding novel and developmentally regulated genes in several species of parasitic protozoa (Chakrabarti et al., 1994; Ajioka et al., 1998; Wan et al., 1999; Li et al., 2003). EST projects in *E. tenella* have been carried out using complementary DNA (cDNAs) from sporozoites and merozoites (Wan et al., 1999; Ng et al., 2002; Li et al., 2003). These stages represent the invasive and intracellular forms of the parasite and are associated with protective immune responses mounted by the host (Rose and Hesketh, 1976; Jenkins et al., 1991; Yun et al., 2000). Currently, very little is known about genes expressed during the remainder of the *Eimeria* life cycle. The aim of this study is to examine genes expressed by oocysts. Unsporulated oocysts shed in feces by chickens are not infectious. Once shed, the oocysts undergo sporulation, a process that culminates in the production of infectious sporulated oocysts containing 4 sporocysts, which in turn contain 2 sporozoites. Because no previous study has concentrated on gene expression by oocysts, it is likely that the majority of the transcripts identified will be novel.

One of the complications of gene discovery through EST analysis is the high probability of repeatedly cloning sequences of highly expressed genes. To avoid this, we attempted to enrich for genes expressed at moderate to low levels by screening

libraries made from *E. tenella* oocysts using a polymerase chain reaction (PCR)–based cDNA subtraction method (Diatchenko et al., 1996). Here, we report the analysis of 499 ESTs isolated from cDNAs enriched for transcripts present in unsporulated and sporulated oocysts through subtractive hybridization. In addition, we investigated the relative expression of 4 transcripts identified by screening the subtracted cDNAs to confirm their differential expression.

MATERIALS AND METHODS

Host infection and parasite recovery

A total of one hundred and eight 4- to 5-wk-old broiler chickens (Moyer's Hatcheries Inc., Quakertown, Pennsylvania) were infected with 1.0×10^5 to 1.25×10^5 oocysts of the Wampler strain of *E. tenella*, mixed in feed. On the seventh day after inoculation, the birds were killed by cervical dislocation and the ceca were removed. The oocysts were recovered as described by Fetterer and Barfield (2003). After cleanup, the oocyst mixture was divided into half. The unsporulated oocysts recovered in one half of the mixture were pelleted and snap-frozen at -70°C , whereas the oocysts in the other half of the sample were sporulated in 2% wt/v potassium dichromate using the protocol described by Fetterer and Barfield (2003). After sporulation, the oocysts were pelleted and snap-frozen at -70°C .

RNA isolation and analysis

Total RNA was isolated from approximately 5×10^6 sporulated and unsporulated oocysts each using TRIzol (Invitrogen, Carlsbad, California). In brief, the sporulated or unsporulated oocysts were combined with approximately 3 g of Pyrex beads (3 mm diameter) (Corning, Corning, New York) and 10 ml of TRIzol. Samples were vortexed for 1 min and incubated on ice for 1 min (4 \times). The oocyst suspension was transferred to a 50-ml round bottom centrifuge tube and 2 ml of chloroform was added. The remainder of the total RNA isolation procedure was carried out according to the manufacturer's recommended protocol. RNA pellets were resuspended in deoxyribonuclease (Dnase)- or ribonuclease-free water (Invitrogen) and placed at -70°C until messenger RNA isolation. To ensure quality, 40 μg of total RNA was run on a 1.5% agarose gel. Poly(A)⁺ RNA was isolated using the MicroFast track kit (Invitrogen), following manufacturer's recommended protocol.

Preparation of cDNA

Both cDNA strands were synthesized from 2 μg of Poly(A)⁺ RNA isolated from sporulated and unsporulated oocysts using polymerases supplied in the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, California). The resulting cDNA was purified by extraction with phenol–chloroform–isoamyl alcohol (24:24:1) (Invitrogen), followed by digestion with *RsaI* for 2 hr at 37°C .

Construction of subtracted cDNA libraries from *E. tenella* oocysts

Subtractions were performed in 2 directions for each tester–driver pair. For the forward subtraction, cDNA from unsporulated oocysts was used as tester and an excess of cDNA from sporulated oocysts was used as driver. This subtraction was used to enrich for differentially expressed transcripts present in unsporulated oocysts. In the reverse subtraction, cDNA from sporulated oocysts was used as tester and an excess of cDNA from unsporulated oocysts was used as driver. The reverse subtraction was used to enrich for differentially expressed transcripts present in sporulated oocysts. Suppression subtractive hybridizations were carried out using the PCR-Select cDNA Subtraction Kit according to manufacturer's recommended protocol.

The 2 pools of subtracted cDNAs, i.e., enriched for transcripts differentially expressed by unsporulated or sporulated oocysts, were amplified by nested PCR using manufacturer's recommended protocol. The PCR products were cloned into pCR2.1 vector (Invitrogen) and were transformed into One Shot TOP10F' competent cells (Invitrogen).

DNA sequencing and analysis

Random clones were picked from Luria Broth (LB) plates containing ampicillin. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, California). Presence of inserts was determined by digesting plasmid DNA with *EcoRI* and electrophoresing digests on 1% agarose gel. Clones containing inserts were sequenced with Universal M13 Forward and Reverse primers. All sequencing reactions were performed using the Big Dye sequencing kit version 3.0 or 3.1 (Perkin–Elmer, Foster City, California) with nonisotopic dye terminators and analyzed on an automated sequencer (Perkin–Elmer ABI PRISM 377 DNA Sequencer). Vector and unreliable sequence was identified using Sequencher 4.1 software (Gene Codes Corporation, Ann Arbor, Michigan). To identify the overlapping sequences, all clones were compared using the “assemble contigs” function of Sequencher 4.1. All clones were compared with sequences in the GenBank database using the Blastn and Blastx algorithms (Altschul et al., 1990). Each unique sequence was compared with the partially completed *E. tenella* genome available at the Sanger Institute (<http://www.sanger.ac.uk/cgi-bin/blast/submitblast/e.tenella/omni>), using WU Blastn. Each unique sequence was also compared with all previously sequenced *E. tenella* ESTs deposited in the dbEST database. The nucleotide sequence data reported in this study has been submitted to EMBL or GenBank database and has been assigned the accession numbers CF983493–CF983716 and CK086213–CK086487.

Quantitative real-time reverse transcriptase–polymerase chain reaction

To validate the differential expression of a portion of the isolated transcripts, real-time quantitative PCR (qPCR) was performed. cDNA templates were generated by reverse transcribing 1 µg of total RNA from sporulated or unsporulated oocysts with random hexamer primers using the Advantage RT-PCR Kit (Clontech). All RNA samples were treated with DNase I (Invitrogen) before cDNA synthesis. Primers were designed manually to amplify approximately 150 bp of transcripts encoding the rhomboid, serpin, prolyl endopeptidase, and protein kinase 5 genes. A fragment of the *E. tenella* small subunit ribosomal RNA (SSU rRNA) was used as a control. Each reaction was carried out in triplicate using the Brilliant SYBR Green Kit (Stratagene, La Jolla, California). The Mx3000p system (Stratagene) was used in generating and detecting fluorescently labeled products. The expression of each transcript was normalized to SSU rRNA using the Q-gene program (Muller et al., 2002).

RESULTS

Screening of cDNAs enriched for transcripts expressed by sporulated and unsporulated oocysts

A total of 225 clones were generated by screening amplified cDNAs enriched for transcripts expressed by sporulated oocysts, whereas 274 clones were generated by screening amplified cDNAs enriched for transcripts expressed by unsporulated

oocysts. Because all cDNAs were digested with *RsaI* before library construction, most of the inserts were fairly small (109–653 bp) and did not contain entire open reading frames. To determine the number of unique sequences, all sequences were compared with one another. This comparison resulted in the formation of 163 unique contigs (Table I). The first 11 sequences are composed of ESTs isolated from cDNA of both sporulated and unsporulated oocysts. The consensus sequence of each contig was compared with the entries in GenBank, and 58 (36%) of the sequences shared significant identity (with a score of at least 1×10^{-4}) with previously described or hypothetical proteins. To ensure that these sequences were not derived from the contaminating host or bacterial DNA, each consensus sequence was compared with the *E. tenella* genome. All but 1 (contig 127) of the sequences matched a segment of the *E. tenella* genome. It is possible that contig 127 represents a sequence of viral origin because a Blastx search revealed that this sequence shares significant (6×10^{-18}) homology with a hypothetical protein from *Burkholderia cepacia* phage. These results indicate that 162 nonredundant sequences presented in this study are encoded in the *E. tenella* genome. To determine whether any novel ESTs were isolated, each consensus sequence was compared with the 28,550 *E. tenella* ESTs derived from cDNA of sporozoites and merozoites. The results from this comparison are shown in column 5 of Table I. A positive match was determined when the overlapping sequences shared at least 95% nucleotide identity. The accession numbers and developmental stage of origin of previously identified ESTs which share the greatest sequence homology to ESTs generated in this study are indicated. If a match occurred between ESTs derived from merozoites and sporozoites, the accession numbers of the highest matching ESTs from each stage are shown. Overall, 36% of the unique sequences overlapped with the previously isolated *E. tenella* ESTs. Categorically, 46% of the sequences from sporulated oocysts and 21% of the sequences from unsporulated oocysts overlapped with known *E. tenella* ESTs.

Highly abundant ESTs

To determine which of the isolated transcripts are most highly expressed, the contigs containing the greatest number of overlapping ESTs were examined. Contigs containing the greatest number of ESTs are: 113, 125, and 153 (Table I). They contain 38, 31, and 16 overlapping ESTs, respectively. All 3 contigs are derived solely from the cDNA of unsporulated oocysts. Contig 113 shares significant identity (7×10^{-23}) with a hypothetical protein of *Plasmodium falciparum*. Contigs 125 and 153 contain sequences from genes that are not similar to any of the previously described genes or proteins. Contig 23 contains partial sequence encoding a microneme protein, Etmic-1, which is important for parasite invasion (Tomley et al., 1991). This contig is composed of 15 ESTs, making it 1 of the most EST-rich contigs derived solely from sequences of sporulated oocysts. Overall, more redundancy was found in ESTs generated from cDNA of unsporulated oocysts. The redundancy of the library constructed from sporulated oocysts was calculated to be 52%, whereas the library constructed from unsporulated oocysts was found to contain 75% redundancy.

TABLE I. List of all unique contigs isolated from cDNAs of sporulated or unsporulated oocysts.

Contig	ESTs per contig	Similarity (Putative ID)*	cDNA source†	Comparison with other <i>Eimeria tenella</i> ESTs
1	2	Putative phosphoethanolamine <i>N</i> -methyltransferase (<i>Arabidopsis thaliana</i> AAM91745)	UO, SO	No match
2	2	Rhomboid family (<i>Plasmodium yoelii yoelii</i> EAA20667.1)	UO, SO	No match
3	4	Transhydrogenase (<i>E. tenella</i> AAA29081.1)	UO, SO	No match
4	11	Eimepsin (<i>E. tenella</i> CAC20153)	UO, SO	No match
5	5	Unknown	UO, SO	Mz§ (BM306346)
6	9	Unknown	UO, SO	Mz (AI676591); Spz (CD661469)
7	2	Unknown	UO, SO	No match
8	4	Unknown	UO, SO	No match
9	3	Hyp. prot. (<i>Plasmodium falciparum</i> NP_701417.1)	UO, SO	No match
10	3	Patched family protein (<i>P. falciparum</i> NP_703278.1)	UO, SO	No match
11	3	Unknown	UO, SO	Mz (AI756383); Spz (CB968491)
12	1	Hsp100 (<i>Trypanosoma cruzi</i> CAA03903.1)	SO	Spz (CF103706)
13	2	Unknown	SO	Spz (BG724483)
14	8	Unknown	SO	No match
15	13	Unknown	SO	No match
16	4	Unknown	SO	Spz (CF103515)
17	2	Stt3 (<i>Toxoplasma gondii</i> CAB38944.1)	SO	No match
18	3	Unknown	SO	No match
19	1	Unknown	SO	No match
20	1	Unknown	SO	No match
21	1	Unknown	SO	Spz (AI757927)
22	4	Unknown	SO	No match
23	15	Microneme-1 (<i>E. tenella</i> AF032905)	SO	Mz (BE028265); Spz (CD346948)
24	4	Unknown	SO	No match
25	1	Unknown	SO	No match
26	1	Unknown	SO	No match
27	2	Unknown	SO	No match
28	2	rabGDI protein (<i>P. falciparum</i> NP_701772.1)	SO	No match
29	2	Microneme-2 (<i>E. tenella</i> Z71755)	SO	Mz (BM305637); Spz (CD666624)
30	2	Unknown	SO	Spz (CF103598)
31	2	Unknown	SO	No match
32	1	GDP-fucose transporter 1 (<i>Dictyostelium discoideum</i> AAO50954)	SO	No match
33	1	Putative Rab7 GTPase (<i>P. falciparum</i> CAB92946.2)	SO	No match
34	3	Sporulated oocyst TA4 antigen (<i>E. tenella</i> P13399)	SO	Spz (CF103987)
35	2	Hsp90 (<i>E. tenella</i> AF042329)	SO	Mz (AI676691); Spz (CD347124)
36	4	Unknown	SO	Mz (AI757700); Spz (CD666530)
37	5	Putative serpin (<i>Echinococcus multilocularis</i> CAD12372.1)	SO	Mz (BM306282); Spz (CD660768)
38	1	Glucosamine fructose 6-phosphate aminotransferase (<i>Plasmodium yoelii yoelii</i> EAA20418.1)	SO	No match
39	1	Aminopeptidase (<i>Arabidopsis thaliana</i> AAN72085.1)	SO	No match
40	1	Hyp. prot. (<i>Neurospora crassa</i> XP_331039.1)	SO	No match
41	6	Unknown	SO	Mz (BM321930); Spz (CD665436)
42	5	Hyp. prot. (<i>P. falciparum</i> NP_700746.1)	SO	No match
43	2	Unknown	SO	No match
44	4	Unknown	SO	No match
45	1	Unknown	SO	No match
46	1	Unknown	SO	No match
47	1	Unknown	SO	Spz (CD664738)
48	1	Unknown	SO	No match
49	1	Unknown	SO	Spz (BG724580)
50	1	Hyp. prot. (<i>P. falciparum</i> NP_700746.1)	SO	No match

TABLE I. Continued.

Contig	ESTs per contig	Similarity (Putative ID)*	cDNA source†	Comparison with other <i>Eimeria tenella</i> ESTs
51	1	Putative tetrapyrrole methylase (<i>Cryptosporidium parvum</i> CAD98667.1)	SO	Spz (CD344042)
52	1	Unknown	SO	Spz (CD659055)
53	1	Unknown	SO	No match
54	1	Unknown	SO	Spz (BG930056)
55	1	Unknown	SO	No match
56	1	Dynein light chain 1, cytoplasmic (<i>P. yoelii yoelii</i> EAA21429.1)	SO	No match
57	1	Unknown	SO	Spz (BG466658)
58	1	<i>E. tenella</i> apicoplast genome (AY217738.1)	SO	Spz (CD657978)
59	1	Unknown	SO	No match
60	1	Unknown	SO	No match
61	1	Unknown	SO	Mz (BM306654); Spz (CD666852)
62	1	Unknown	SO	No match
63	1	Unknown	SO	No match
64	1	Unknown	SO	Spz (CF104096)
65	1	Unknown	SO	Sporozoite (BG466724)
66	1	Unknown	SO	Spz (BG516326)
67	1	Unknown	SO	No match
68	1	<i>Eimeria bovis</i> developmental mRNA (M98842)	SO	No match
69	1	Subtilisin-like serine protease (<i>Nostoc punctiforme</i> ZP.00107311.1)	SO	No match
70	1	Unknown	SO	Mz (AI676662); Spz (CD344061)
71	1	<i>E. bovis</i> developmental mRNA (M98836.1)	SO	No match
72	1	Unknown	SO	No match
73	1	Unknown	SO	No match
74	1	Unknown	SO	Spz (BG466769)
75	1	Unknown	SO	Spz (CD665461)
76	13	Unknown	SO	Spz (BG724995)
77	3	Unknown	SO	No match
78	1	Unknown	SO	Spz (AI759572)
79	1	Unknown	SO	No match
80	1	Unknown	SO	No match
81	6	Unknown	SO	No match
82	2	Microneme-5 (<i>E. tenella</i> ETE245536)	SO	Mz (BM306798); Spz (CD346269)
83	2	Unknown	SO	Mz (AI759292); Spz (CF104123)
84	1	Unknown	SO	No match
85	1	Hyp. protein (<i>P. falciparum</i> NP_703489.1)	SO	Mz (BM305947); Spz (CD666502)
86	1	Unknown	SO	Spz (BG930136)
87	2	EF-Tu family (<i>C. parvum</i> CAD98437.1)	SO	Spz (BG929822)
88	1	Unknown	SO	No match
89	2	Putative cGMP phosphodiesterase A4 (<i>P. yoelii yoelii</i> EAA15787.1)	SO	No match
90	2	Hsp90 (<i>E. tenella</i> AF042329)	SO	Mz (AI676522)
91	2	Unknown	SO	Spz (CD665059)
92	1	Unknown	SO	Mz (BE028723); Spz (CD346117)
93	1	Unknown	SO	No match
94	1	Ubiquitin-conjugating enzyme (<i>P. yoelii yoelii</i> EAA21159.1)	SO	Spz (CF103872)
95	1	Unknown	SO	Mz (BM306118); Spz (CF103878)
96	1	Hyp. prot. (<i>P. falciparum</i> NP_705850.1)	SO	Mz (BM306336)
97	3	Unknown	SO	Spz (CB968334)
98	1	Unknown	SO	No match
99	1	Phosphate dep. phosphofructokinase (<i>P. yoelii yoelii</i> EAA20618.1)	SO	No match
100	1	TA4 antigen (<i>E. tenella</i> P13399)	SO	Spz (CD569482)
101	1	Glyoxase II (<i>A. thaliana</i> CAA69644.1)	SO	No match

TABLE I. Continued.

Contig	ESTs per contig	Similarity (Putative ID)*	cDNA source†	Comparison with other <i>Eimeria tenella</i> ESTs
102	1	Unknown	SO	Mz (AI757327); Spz (CD658167)
103	1	Apurinic/Apyridinic endonuclease (<i>P. falciparum</i> AF284763.1)	SO	No match
104	1	Unknown	SO	Mz (BE027218); Spz (BG930070)
105	1	Unknown	SO	No match
106	1	Unknown	SO	Mz (BE027796); Spz (CD666323)
107	4	Nam-7 protein (<i>P. yoelii yoelii</i> EAA17820.1)	UO	No match
108	2	Unknown	UO	No match
109	1	Putative snRNP (<i>Sorghum bicolor</i> AAM94328.1)	UO	No match
110	1	Unknown	UO	No match
111	1	Unknown	UO	No match
112	3	Actin (<i>P. falciparum</i> NP_701803.1)	UO	Mz (AI676590); Spz (CD659971)
113	38	Hyp. prot. (<i>P. falciparum</i> NP_703524.1)	UO	No match
114	2	Insulin degrading protein (<i>Caenorhabditis elegans</i> NP_507226.1)	UO	No match
115	1	Putative, glycerol-3-phosphate dehydrogenase (<i>P. falciparum</i> NP_701017.1)	UO	No match
116	15	Unknown	UO	No match
117	4	Interferon, gamma-inducible protein 30 (<i>Homo sapiens</i>)	UO	Spz (BG516289)
118	2	Unknown	UO	No match
119	2	Unknown	UO	No match
120	2	Unknown	UO	No match
121	15	Putative prolyl endopeptidase (<i>A. thaliana</i> AC009978.3)	UO	No match
122	1	Unknown	UO	No match
123	3	Unknown	UO	No match
124	3	Unknown	UO	No match
125	31	Unknown	UO	No match
126	4	Hyp. prot. (<i>P. yoelii yoelii</i> EAA15481.1)	UO	Spz (BG930053)
127	2	Hyp. prot. Bcep22p33 (<i>Burkholderia cepacia</i> phage NP_944263)	UO	No match
128	2	Unknown	UO	No match
129	1	Unknown	UO	Mz (BE027701)
130	1	Hyp. prot. (<i>Anopheles gambiae</i> XP_310419.1)	UO	No match
131	4	Unknown	UO	No match
132	6	Unknown	UO	No match
133	1	Unknown	UO	No match
134	1	Unknown	UO	No match
135	3	Histone H2A.F/Z (<i>T. gondii</i> AF502246.1)	UO	No match
136	1	Putative cAMP-dependent protein kinase (<i>P. yoelii yoelii</i> EAA23025.1)	UO	Mz (AI676708)
137	1	Unknown	UO	No match
138	1	TA4 ag (<i>E. tenella</i> AAA29074.1)	UO	Spz (CF104098)
139	2	23S rRNA gene; plastid (AF307888 <i>E. tenella</i>)	UO	Mz (BI895110); Spz (CD666240)
140	1	Unknown	UO	No match
141	1	Unknown	UO	No match
142	1	Transhydrogenase (<i>E. tenella</i> T18520)	UO	No match
143	4	Protein kinase 5 (<i>T. gondii</i> AAD17246.1)	UO	No match
144	2	Unknown	UO	No match
145	2	Unknown	UO	No match
146	3	BT1 family protein (<i>C. parvum</i> CAD98492.1)	UO	No match
147	3	Hyp. prot. (<i>N. crassa</i> XP_327161.1)	UO	Mz (AI756406); Spz (CD660357)
148	2	Unknown	UO	Spz (BG561905)
149	3	F-LANa (<i>H. sapiens</i> AF208065.1)	UO	Mz (BM306589); Spz (CD347073)
150	3	Unknown	UO	Mz (AI756014); Spz (BG235865)
151	13	Unknown	UO	No match
152	9	Unknown	UO	No match
153	16	Unknown	UO	No match

TABLE I. Continued.

Contig	ESTs per contig	Similarity (Putative ID)*	cDNA source†	Comparison with other <i>Eimeria tenella</i> ESTs
154	9	Unknown	UO	No match
155	1	Unknown	UO	No match
156	14	Unknown	UO	No match
157	1	Unknown	UO	No match
158	1	Unknown	UO	No match
159	1	Transhydrogenase (<i>E. tenella</i> T18520)	UO	Spz (CD660857)
160	1	Hyp. prot. (<i>P. falciparum</i> NP_701032.1)	UO	No match
161	1	Unknown	UO	No match
162	1	Unknown	UO	No match
163	1	Unknown	UO	No match

* Contigs were assigned a putative identity when a score of at least 1×10^{-4} was obtained by comparing with previously described proteins using Blastx. When putative homology was found, the name of the highest scoring sequence, the organism from which the sequence originated, and the accession number (in parentheses) are listed in the column below.

† The sequences reported here are derived from sporulated oocysts (SO), unsporulated oocysts (UO), or both (UO, SO).

‡ Contigs were assigned a match between previously isolated *E. tenella* ESTs when at least 95% nucleotide identity was shared between the query and subject. The dbEST accession numbers are shown in parentheses for ESTs which shared the highest level of nucleotide identity with the query and the stage of the life cycle from which they were derived.

§ Mz, merozoites.

|| Spz, sporozoites.

Quantitative real-time PCR

To determine whether subtractive hybridization is a suitable method for enriching for differentially expressed genes, expression levels of 4 genes isolated during this study were determined using real-time qPCR. All 4 genes appear to be differentially expressed between unsporulated and sporulated oocysts (Fig. 1). ESTs encoding a serpin were isolated from cDNA of sporulated oocysts. qPCR confirmed that serpin transcripts are expressed by sporulated oocysts but are undetectable in cDNA of unsporulated oocysts (Fig. 1A). Expression of 2 genes

(prolyl endopeptidase and protein kinase 5) isolated only from cDNA of unsporulated oocysts was investigated. Transcripts of both genes were readily detectable in cDNA from unsporulated oocysts, but their expression in sporulated oocysts was approximately 10 times less (Fig. 1C, D). ESTs encoding the rhomboid gene were isolated from both sporulated and unsporulated oocysts, and although these transcripts were identified from both the cDNA pools, qPCR indicated that this gene is also differentially expressed, with greater expression associated with unsporulated oocysts (Fig. 1B).

DISCUSSION

Sequencing ESTs from apicomplexan parasites has in the past been proven to be a useful yet inexpensive tool in gene discovery as well as in expression studies (Chakrabarti et al., 1994; Ajioka et al., 1998; Wan et al., 1999; Li et al., 2003). In species for which complete genome sequences are available, ESTs have proven valuable in determining the positions and structures of genes (Gardner et al., 2002). The 499 sequences from *E. tenella* presented here represent the first EST analysis from *Eimeria* oocysts. In the past, most, if not all, EST studies (for example: Wan et al., 1999; Ng et al., 2002; Li et al., 2003) carried out in *E. tenella* have focused on describing transcripts from merozoites and sporozoites. This is a useful approach because these 2 stages ultimately lead to the formation of the large maturing schizonts that are responsible for most of the pathology in the host. However, the oocysts should not be overlooked because these developmental stages are responsible for parasite dispersal and subsequent host entry. The number of ESTs analyzed in the study is small, nonetheless, it is apparent that most of the transcripts expressed by either sporulated or unsporulated oocysts represent novel genes that have not been previously isolated from other organisms. It is not altogether surprising that most of the transcripts encode unknown genes because from the complete genome sequence of *P. falciparum*, it was deduced

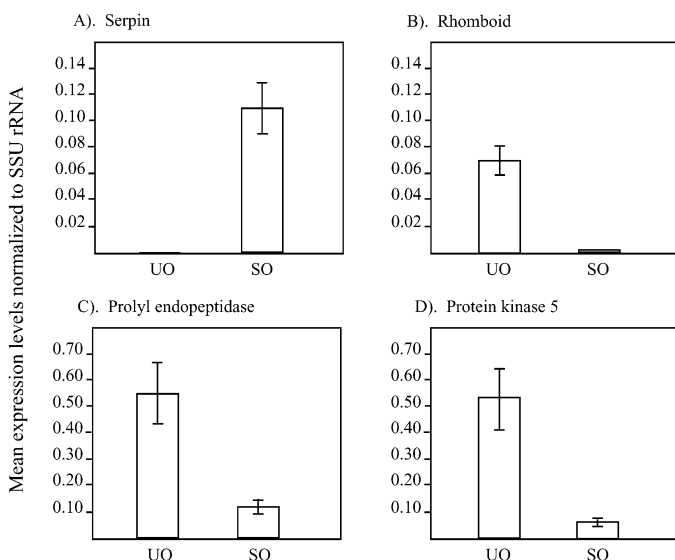


FIGURE 1. Normalized expression levels of 4 transcripts, (A) serpin, (B) rhomboid, (C) prolyl endopeptidase, and (D) protein kinase 5, isolated from subtracted cDNA libraries. All 4 transcripts were found to be differentially expressed between sporulated and unsporulated oocysts.

that approximately 60% of all predicted proteins did not share significant sequence identity with proteins from other organisms (Gardner et al., 2002). Therefore, most apicomplexan genes do not have homologs in the genomes of members of other phyla.

To avoid isolating predominantly housekeeping genes such as ribosomal proteins, the cDNAs from sporulated and unsporulated oocysts used in library construction were subtractively hybridized with each other. Theoretically, these subtracted cDNAs should be enriched for rare transcripts that are differentially expressed. Expression of 4 transcripts found in the course of this study was investigated using qPCR, and the results indicate that these genes are differentially expressed between sporulated and unsporulated oocysts. These data suggest that subtractive hybridization can be used to enrich for developmentally regulated transcripts. In addition, no transcript encoding ribosomal proteins (which are often thought to represent housekeeping proteins) was found, indicating that these transcripts have been successfully subtracted out.

By comparing ESTs presented in this study with previously isolated ESTs from *E. tenella* merozoites and sporozoites, we found that almost half of the nonredundant sequences isolated from sporulated oocysts have been previously isolated from either sporozoites, merozoites, or both. It is not surprising that sporulated oocysts and sporozoites share many of the same transcripts because essentially the sporulated oocyst contains 8 sporozoites. Even though many transcripts are shared between these 2 stages, more than half of the sequences transcribed by sporulated oocysts have not been previously isolated from either merozoites or sporozoites and may represent genes whose expression may be limited to the sporulated oocyst. In contrast to genes expressed by sporulated oocysts, the vast majority of transcripts expressed by unsporulated oocysts have not been previously isolated from either sporozoites or merozoites. Once again this is not altogether surprising because unsporulated oocysts represent a fertilized, undifferentiated stage of the life cycle, which is very different from the highly differentiated, invasive sporozoites and merozoites, morphologically as well as physiologically.

One of the most abundant transcripts isolated from sporulated oocysts encodes the microneme protein, Etmic-1, which accounts for 6.7% of all sequences recovered from sporulated oocysts. Two other transcripts that encode microneme proteins 2 and 5 were also isolated; however, these account for only 0.9% of all sequences isolated from sporulated oocysts. Microneme proteins are localized to the microneme, an organelle that plays an important role in parasite invasion (Tomley and Soldati, 2001). In previous studies, expression of microneme genes was detected from hour 12 of sporulation and was maintained through 48 hr; however, transcripts were not detected in unsporulated oocysts (Ryan et al., 2000). This analysis supports this data because transcripts encoding 3 different microneme genes were detected only in cDNA of sporulated oocysts. The most abundant transcripts isolated from unsporulated oocysts represent genes of unknown function, which have not been previously isolated from either merozoites or sporozoites. Only further characterization of these genes and their products will enable us to determine their roles in oocyst function.

Altogether, we found that subtractive hybridization can be used in identifying transcripts whose expression is differentially regulated in 2 distinct pools of cDNA. In addition, this method

may be useful in identifying transcripts whose expression has not yet been detected through the standard EST screening.

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